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THE RELATION OF THE PSEUDODIPHTHERIA AND THE DIPHTHERIA BACILLUS.*

Paul F. Clark.

(From the Bacteriological Laboratory of Brown University.)

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INTRODUCTION.

WITH the advance of our knowledge of the bacteria, both pathogenic and non-pathogenic, numerous organisms have been discovered similar to the normal type in most respects, but differing from it in certain characters. Bacteria are extremely variable organisms, altho the limits to this variability seem to be more sharply defined than was formerly supposed. This variability depends largely upon two main factors. The first is the enormous number of generations which may develop in a short period of time. With the bacteria it is indeed true that "one day is as a thousand years." The second is the fact that because of the simple single-celled structure of bacteria, acquired characters can, to a certain extent, be inherited. The effect of the environment is, therefore, of great importance in any study of bacteria. Variations, caused by the nature of the conditions under which organisms have recently been living, must be considered as well as the ordinary fluctuating variations and mutations found in all forms of plants and animals. It is because of these facts that the idea of "groups" among the bacteria has developed in recent years. Within each group are found many different types or species which add greatly to the difficulties of identification and present puzzling problems as to the nature of the interrelationship among the several forms.

The group of diphtheria organisms exhibits, possibly more than any other, a greater variety of these somewhat similar types. Löffler himself discovered the so-called pseudodiphtheria bacillus in 1887 and a few months later it was independently observed by Hofmann-Wellenhof (1888). B. xerosis was described by Kutschbert and Neisser (1884) and since that time a large number of other somewhat similar types, known generally as diphtheroids, have added to the general interest and confusion. In this group, also, the problem of relationship is of special importance because of its bearing on the bacteriological control of contacts, carrier cases, quarantine, treatment of epidemics, and other public-health methods.

There have been, in general, two theories held by the different observers in regard to relationships of the different organisms of this group. On the one hand, we find such men as Roux, Yersin, Behring, Wesbrook, and others upholding the view that all of these types are variants of the same species. On the other hand, we find by far the greater number of bacteriologists, including Löffler, Escherich, Fränkel, Flügge, etc., believing that at least the first three forms mentioned are three distinct species, while opinion seems to be about evenly divided as to the classification of the diphtheroids.

Particularly among the earlier writers, considerable confusion existed as to the exact meaning of the term pseudodiphtheria bacillus. The descriptions of both Hofmann and Löffler in regard to this organism are rather indefinite. There can be little doubt that many of the discrepancies apparent in the literature are due to the inaccuracy of our definitions and that many of the writers have been dealing with atypical Klebs-Löffler bacilli.

In later years, there has been a tendency to distinguish between the different organisms by the difference in pathogenicity and in their action in dextrose broth. Park and Beebe's (1895) classification based on these points, is as follows:

I. Bacilli identical in appearance, both in culture and under the microscope, with the diphtheria bacillus.
    a) Pathogenic acid producers equal virulent diphtheria bacilli.
    b) Non-pathogenic acid producers equal non-virulent bacilli.
II. Bacilli somewhat resembling, but shorter and stouter than diphtheria bacilli.
    a) Non-pathogenic, non-acid producers equal Hofmann's or the pseudodiphtheria bacilli.

The use of this scheme and also Wesbrook's more elaborate classification has given greater accuracy to the observations of the more recent workers in this field.

REVIEW AND DISCUSSION OF PREVIOUS WORK ON THE SUBJECT.

The distinctive features generally used for purposes of differentiation between the various members of this group are: 1. *Cultural characteristics.* 2. *Staining reactions.* 3. *Action upon carbohydrates.* 4. *Serum reactions.* 5. *Morphology.* 6. *Pathogenicity.*

The first two of these may be dismissed very briefly as practically all recent observers admit that, while these points are of service generally, they are not sufficiently constant to be made the criterion of specific difference. It is generally recognized that B. diphtheriae varies greatly on the different culture media and therefore no reliance can be placed upon that feature. Even the double stains of Neisser, Falieres, Ljubinsky, and others are unreliable, and many observers prefer the ordinary Löffler's stain which is simpler and gives exactly as much information as any of the more complicated processes.

The other characteristics are of more importance, however, and it may be well to review briefly some of the more recent researches upon these points.

Action upon carbohydrates.—Roux and Yersin (1888) appear to be the first to have noticed that the diphtheria bacillus produced acid in broth and most subsequent observers have agreed with them. Zarniko (1889) observed that Hofmann's bacillus produced in broth cultures either an alkaline reaction or at first a weakly acid followed by an alkaline reaction. Escherich (1894) went so far as to declare that acid production was proof positive of the pathogenicity of a given strain.

Spronck (1895) pointed out that if diphtheria organisms are grown in dextrose-free broth, the reaction remains alkaline; if small quantities of the muscle sugar are present, the reaction becomes at first acid and later alkaline; while if much dextrose is added, the broth attains an acid reaction which is permanent. Numerous observers have confirmed these results.

Less work has been done on the other sugars, but according to L. Martin (1898) the diphtheria bacillus produces acid from dextrose, levulose, galactose, saccharose, and glycerin, but not from maltose, lactose, mannit, arabinose, raffinose, dulcite, glycogen, erythrite, and starch. Practically all other writers differ with him, however, as regards the action on saccharose.

More recently Knapp (1904), in testing 27 cultures of diphtheria bacillus, 10 of xerosis bacillus, and 4 of pseudodiphtheria bacillus, came to the conclusion that the action in saccharose and dextrin media will differentiate between these three species, as follows:

The pseudodiphtheria bacillus ferments neither sugar.

The xerosis bacillus ferments saccharose.

The true Klebs-Löffler bacillus ferments dextrin.

Hamilton and Horton (1906) were unable to confirm Knapp's work except as regards dextrin.

Graham-Smith (1906) describes experiments on 23 cultures of diphtheria bacilli (18 fully virulent, 5 non-virulent), 20 cultures of Hofmann's bacilli, 3 cultures of xerosis bacilli, and some other diphtheria-like bacilli. He used mostly the serum medium of Hiss with an incubation of 10 days and concludes from this work that some of the contradictory results obtained by various investigators are due to the fact that many strains of organisms will not grow well in broth when first isolated from the throat. His tests show that under suitable conditions all strains of diphtheria bacilli produce acid from glucose, galactose, levulose, and maltose and the majority from dextrin and glycerin. The action on lactose is very variable and only a few strains act on saccharose. Hofmann's bacillus produced acid in no case. The xerosis bacillus produced a small amount of acid from glucose, levulose, and glycerin, and a still smaller amount from saccharose. Zinsser (1907) working with 42 strains of B. diphtheriae, 21 strains of B. xerosis, and 8 strains of B. hofmanni confirmed Knapp's results. Goodman (1908) examined the action of 103 strains of Klebs-Löffler bacilli on dextrose, dextrin, maltose, and saccharose. By titration, he found a wide range of variation in the amount of acid produced, that from dextrose extending from +0.1 to +4.0. From one strain, he made a series of artificial selections, choosing each time the highest and lowest acid producer. Starting with +2.3 and +1.9 as the high and low cultures, after 36 selections, he obtained +4.4 and −0.5 as the greatest and the least acid producers respectively. The morphology of these last two cultures was still similar to that of the parent culture. The pathogenicity, however, was not satisfactorily tested. He concludes that the division of the diphtheria group into several distinct species is probably based on a misconception.

Serum reactions.—Spronck (1896) describes a test in which he inoculates a guinea-pig with antitoxin and then with the organism to be examined. Complete protection against the organism denotes it to be the true diphtheria bacillus; local edema only, as in the controls, denotes the pseudodiphtheria bacillus. This test is, of course, valueless with wholly non-virulent organisms. C. Fränkel (1897) confirms these results. Bergey (1898) could produce no immunity against the Klebs-Löffler bacillus by several inoculations of Hofmann's bacillus. Lubowski (1900) immunized a goat by inoculating with non-virulent diphtheria bacilli. The serum thus obtained agglutinated 23 strains of typical diphtheria organisms and two of non-virulent organisms at a dilution of 1:80. B. hofmanni and cocci were not agglutinated.

Lesieur (1901) immunized a horse against Klebs-Löffler bacilli and obtained a serum which agglutinated some strains of diphtheria organisms but not others. It acted in a similar fashion toward the pseudodiphtheria bacilli. Fifty of his 70 strains were not agglutinated.

Schwoner (1902) obtained a serum by injecting a horse first with dead and then with living B. diphtheriae. This agglutinated diphtheria bacilli at a dilution of 1:500 and some strains as high as 1:10,000. It agglutinated pseudodiphtheria organisms at a dilution of 1:10 to 1:40 exactly as in the case of normal and antitoxic sera. He also (1904) shows that cultures of diphtheria bacilli from severe cases exhibit hemolytic power on rabbits' corpuscles. Pseudodiphtheria bacilli do not show this action.

Hamilton and Horton (1906) obtained a serum from goats and rabbits immunized against one strain of their virulent pseudodiphtheria bacilli. This proved to be bactericidal to the other virulent pseudodiphtheria strains but not to non-virulent pseudodiphtheria bacilli nor to true diphtheria bacilli.

Morphology.—Roux and Yersin (1890) discovered no morphological alteration of a strain of B. diphtheriae by growth at 39°5 C. Hewlett and Knight (1897) state that the pseudodiphtheria bacillus seems to replace the typical diphtheria organism during convalescence. They illustrate this with a list of bacteriological examinations of 24 cases. Wesbrook, Wilson, and McDaniel (1900) introduced a classification of the various types of diphtheria organisms based on an extensive study of the morphology in pure cultures. These types are divided into three main groups: granular, barred, and solid-staining forms with numerous subdivisions based upon size and shape. They observe that the granular types usually give place wholly or in part to barred and solid types shortly before the disappearance of diphtheria-like organisms.

Gorham (1901) corroborates this and believes that the change is caused by the effect of the body fluids of persons becoming slowly immune or those entirely non-susceptible. Lesieur (1901), by growth for eight months in diffuse daylight in a dry room, transformed three diphtheria strains to pseudodiphtheria types. By passage in collodion sacs through three rabbits, B. pseudodiphtheriae took on the morphology of B. diphtheriae. He was also able to do this with two out of four strains of the pseudodiphtheria bacillus by repeated transfer in broth, and in one out of three, by a single passage through broth in symbiosis with Aurococcus aureus.

Cobbett (1901) records a series of examinations made during the progress of the disease in a number of cases and came to the conclusion that D^2 (Wesbrook's classification) does not replace typical forms during convalescence. He suggests that a possible cause of observations to this effect might be that in the early stages of the disease the diphtheria organisms are readily found so that a more careful examination is omitted. Later, the diphtheria organisms become fewer and a more vigorous search must be made. In the course of this more thorough examination, naturally more D^2 are met with and a false impression as to their relative prevalence is produced. He also states that D^2 is not more frequently found in the noses and throats of persons in close relation with those sick with diphtheria than in those who apparently have had no contact with the disease:

Ohlmacher (1902) changed a typical diphtheria organism to an organism resembling Hofmann's bacillus by 48 hours' subcutaneous growth in an immune animal, a white rat. He also changed a slightly virulent pseudodiphtheria type to a typical diphtheria form with increased virulence by one passage through a guinea-pig. Denny (1903) made a series of frequent microscopic examinations during the early growth of B. diphtheriae, B. pseudodiphtheriae, and B. xerosis. He found that in young cultures all three present striking similarities showing a large proportion of D^2C^2 types. In older cultures, the true diphtheria and xerosis bacilli displayed developmental differences characteristic of the higher bacteria, such as granulation, segmentation, etc., altho the forms of the xerosis bacilli were usually shorter and thicker than those of the Klebs-Löffler bacilli. Hofmann's bacillus always remained typical in form. He found that unfavorable conditions, such as symbiosis with a large number of other organisms, delayed the formation of granular types and suggests that this may explain the greater abundance of solid forms in the 15-hour cultures as convalescence advances and the proportion of ordinary throat bacteria increases.

Graham-Smith (1903) reports that some B. hofmanni are clubbed and often slightly curved but are broader and take the stain more deeply than B. diphtheriae. They sometimes show stained segments which are very dark and well defined, the septa being narrow and running in all cases transversely across the bacilli. These forms always revert to the typical D^2 type. Smirnow (1908), in using double-walled celloidin sacs to study symbiotic relations of B. diphtheriae with other organisms, recovered coccoid forms from the compartment originally containing the diphtheria bacilli. These reverted to typical form, however, after growth in broth and then on blood-serum.

Pathogenicity.—Löffler considered this the most unvarying feature of the diphtheria organism. The gelatinous edema in the subcutaneous tissue at the point of inoculation, the congestion of various organs, and more especially the hyperemia and enlargement of the suprarenal capsules are certainly highly characteristic. Roux and Yersin (1890) were able to increase the virulence of slightly virulent diphtheria bacilli by injection together with erysipelas streptococci. They were unable to do this with non-virulent organisms, however. Trumpp (1896) transformed an avirulent D^2 type to a typical diphtheria organism by passage through three guinea-pigs together with a non-fatal dose of diphtheria toxin. His organism produced acid, however, and he does not believe it possible thus to transform a non-acid former.

Hewlett and Knight (1897) changed a typical acid-producing virulent diphtheria culture into a non-virulent D^2 by heating for seventeen hours at 45° C. By prolonged cultivation for 20 generations and then long incubation and passage through a guinea-pig they also changed a typical non-acid-producing D^2 into an acid-producing typical diphtheria organism. They were unable to repeat these experiments successfully.

Bergey (1898) was unable to give virulence to D^2 by passage through several animals. Martin (1898) increased the virulence of slightly virulent diphtheria organisms by growth in celloidin sacs in the body cavity of rabbits. Salter (1899) by five successive passages through goldfinches exalted the virulence of four separate strains of typical pseudodiphtheria bacilli to a point where they were capable of killing guinea-pigs. The transformation was complete both as to morphology and acid production. The toxic action was entirely neutralized by diphtheria antitoxin. Nineteen strains of pseudodiphtheria bacillus proved virulent for goldfinches and chaffinches, 18 out of the 19 were virulent for canaries, and many of the strains were virulent for other birds.

Davis (1898) isolated 12 strains of virulent pseudodiphtheria organisms which produced a general bacteremia and against which diphtheria antitoxin afforded no protection. Wesbrook, Wilson, and McDaniel (1900) report an outbreak of diphtheria in which the type most frequently found resembled D^2. They all produced acid, however, in dextrose broth and the toxin formed was neutralized by diphtheria antitoxin. Gorham (1901) says that type D^2 has sometimes proved pathogenic to guinea-pigs. Cobbett (1901) isolated and tested 86 strains of the pseudo-diphtheria bacillus. They all proved to be non-virulent in doses of 2 c.c. of a 48-hour culture. Lesieur (1901), as already noted above under "Morphology," was able to make several strains of pseudodiphtheria bacilli virulent and also accomplished the reverse transformation. Neumann (1902) examined 78 strains of D^2 all of which proved to be non-virulent. Ohlmacher (1902) transformed a slightly virulent D^2 type by one passage through a guinea-pig and at the same time increased its virulence.

Williams (1902) made a careful series of investigations extending over a period of about seven years. Inoculations of large doses of typical D^2 proved to be innocuous to goldfinches. Successive peritoneal inoculations of D^2 produced no exaltation in virulence. Four strains of typical diphtheria bacilli showed no change after growing in an immune host (white rat) for 48 hours. Two non-virulent but morphologically typical strains of diphtheria organisms were grown with virulent streptoccoci in broth for 90 generations, transplanted every three or four days. When separated, no change whatsoever was observed. Two cultures of Klebs-Löffler bacilli were cultivated for several months at 40° C., transplanting them every week, and six cultures were grown for the same length of time at 43° C.—45° C., alternating every week to a temperature of 35° C. These organisms were somewhat smaller at the high temperatures but reverted to their normal size when placed at body heat. The author observed no sequence of types during the course of the disease and considered the morphology of the several species to be quite distinct.

Graham-Smith (1904) observed no partially attenuated diphtheria bacilli, altho 25 strains out of 113 which he examined were entirely non-virulent. Ruediger (1903) and Hamilton (1904) describe three varieties of pseudodiphtheria bacilli, one of which is pathogenic to guinea-pigs. This produces a general bacteremia and is neutralized by the serum of a rabbit immunized against any of the virulent varieties, but not by regular diphtheria antitoxin. Wesbrook (1905) himself states that in dealing with epidemics, bearers of all forms other than A, C, and D may be safely disregarded. Hadley (1907) recovered D^2 forms from inoculations of three strains of virulent C, C^1, and C^2 forms. Also one of his D^2 types proved to be virulent but, when recovered after inoculation, proved to be of the barred type. Corbett (1906) states that D^2, E^2, and C^2 types generally prove to be virulent. Zinsser (1907) found no virulent pseudodiphtheria bacilli.

No exhaustive analysis of this literature is necessary to make clear the fact of the many discrepancies in the results obtained by different observers. The fermentative action of the true diphtheria bacillus upon dextrose and some other carbohydrates and the characteristic toxic action, however, stand out as the features about which there is the least dispute. It is almost universally conceded that B. hofmanni produces little or no acidity in dextrose broth and is usually non-pathogenic to guinea-pigs or at most produces only a slight local edema at the point of inoculation. With the true Klebs-Löffler bacillus, however, the toxic action is peculiarly characteristic and it generally produces acid in dextrose broth.

In view of the contradictory observations and diverse opinions in regard to the relation of the members of this group, the writer determined to investigate further the question as to the identity of the pseudodiphtheria and the diphtheria bacillus. The investigation was not extended to a consideration of B. xerosis and the other diphtheroid bacilli.

STATEMENT OF THE PROBLEM TO BE SOLVED.

The crux of the situation then seems to be: Can a typical non-acid-producing non-pathogenic Hofmann's bacillus be changed into a typical acid-producing pathogenic Klebs-Löffler bacillus whose toxic action can be neutralized by commercial diphtheria antitoxin? And if this is true in the laboratory, does it necessarily hold true in nature? Further, does the reverse transformation take place? Do the varieties of virulent diphtheria change through the course of the disease from the granular to the segmented type, and, becoming less and less virulent, finally take on the form of the non-virulent pseudodiphtheria bacillus?

EVIDENCE BEARING ON THE RELATION OF THE PSEUDODIPHTHERIA AND THE DIPHTHERIA BACILLUS.

From a series of cultures taken during the course of the disease.—A tabulation of the Wesbrook types obtained from smear preparations of cultures was made. These cultures were taken mostly from persons suffering from the disease, altho a few were from contacts. Most of the examinations were made by the writer as part of the routine work in the laboratory of the Rhode Island State Board of Health. Some, principally those taken toward the end of the disease from cases in Providence, were made by Professor F. P. Gorham, bacteriologist of that city. The results are shown in Table 1.

These results, while not sufficient to draw any far-reaching conclusions, seem to point to a decision against the Wesbrook-Gorham theory. Solid-staining C^2 and D^2 forms are found fully as often in the earlier cultures as in those taken when the patient was convalescing. Another point to be noticed is that the same types were not constantly present but appeared to vary considerably from time to time.

In this connection, it is worthy of note that during the last year or two, the organisms from clinical diphtheria cases in Providence have very frequently been barred forms instead of granular types as was formerly the case. If, as some advise, a positive diagnosis should be given only when the granular forms are present, a large percentage of the cases of clinical diphtheria in this vicinity would be overlooked.

From attempts to establish virulence in a non-virulent organism.—The first step was to obtain a number of pure cultures of B. hofmanni. These were isolated, some from cultures sent in to the Rhode Island State Board of Health Laboratory for diagnosis but mostly from cultures from contacts obtained from the City of Providence Laboratory. Following is a list (Table 2) of the different strains isolated with their action on guinea-pigs and reaction to phenolphthalein when grown in one per cent dextrose broth for three days.

Most of these cultures were tested for purity several times before using them. During the first part of this investigation the cultures for inoculation were grown in ordinary nutrient broth. Subsequently, however, they were always grown in dextrose-free broth in order to have the media as favorable as possible for toxin formation. A growth of 48 hours was used in most cases, for inoculation, but occasionally a longer growth was employed, especially when the two-day cultures were not well grown. Smear preparations were in all cases stained with Löffler's methylene blue.

The 23 cultures of typical B. hofmanni (Nos. 1–9 inclusive and 14–27 inclusive) were tested for virulence upon half-grown guinea-pigs. They all proved non-virulent or only slightly virulent (i.e., producing local edema only) in doses of one per cent of the body weight. Most of the strains produced a slight local edema at the point of inoculation and some of the guinea-pigs showed a small loss

TABLE 1.
TABULATION OF SUCCESSIVE CULTURES.
CLINICAL CASES.

Name	First Culture	No. of Days Intervening	Second Culture	No. of Days Intervening	Third Culture	No. of Days Intervening	Fourth Culture	No. of Days Intervening	Fifth Culture	No. of Days Intervening	Sixth Culture
E. S. C.	C^1D^2	1	D^2C^2	5	$D^2C^2A^2$						
W. B.	$A^1C^1C^2$	11	$C^1C^2A^2D^2$								
C. B.	C^2D^2	13	$CC^1C^2D^2$								
E. D.	$C^1C^2D^2$	5	C^2D^2	3	$C^1C^2D^2$						
E. T.	$C^1C^2D^2$	11	D^2	5	D^2						
L. T.	C^1C^2	11	C^2								
L. D.	$C^1C^2D^2$	7	D^2								
H. W.	C^1C^2	5	C^1A^2(few)D^2								
M. D.	C^1C^2	19	CC^2	6	C^2						
P. R. L.	C^2D^2	15	C^1C^2	3	C^1C^2	4	$C^1C^2D^2$				
R. L.	C^1	9	$C^1A^1C^2$								
L. C.	$C^1C^2A^1$	14	$C^1C^2A^1$	8	C^1C^2						
S. C.	$C^1C^2D^2$	5	C^1C^2	3	C^2	1	C^2	6	C^2	2	C^1A^1
								2	$C^1C^2A^1A^2$	2	C^2
R. M.	C^1C^2	16	C^1C^2	6	C^1C^2						
Mrs. B.	$C^1C^2D^2$	16	C^2	1	C^2						
A. C.	C^1	13	C^1C^2	3	C^2D^2						
J. B.	C^1C^2	19	C^1C^2	4	C^2						
M. P.	$C^2D^2C^1$	13	$C^2D^2C^1$								
M. C.	C^2D^2	2	C^1A^1	3	D^2						
J. H.	C^1C^2	4	C^1A^1	4	C^2	3	$C^1C^2D^2A^1$				
J. V. G.	C^2C^1	12	D^2								
F. W.	C^1	20	D^2	5	D^2C^2						
C. M.	$D^2C^2C^1$	3	D^2C^1								
A. B.	CD	6	C^1C^2	3	D^2C^1	2	D^2	2	D^2C^2		

CONTACT CASES.

E. H. W.	D^2 (nose)	2	D^2 (throat)	2	D^2 (nose)	5	D^2 (throat)		D^2 (throat)	2	E^2 (nose)
			D^2C^2 (nose)				D^2 (nose)	3	C^1A^1 (nose)		
W. T. W.	D^2 (nose)	2	D^2C^2 (nose)	4	D^2 (throat)		D^2 (throat)		D^2C^2 (throat)		$D^2C^2C^1$ (throat)
					D^2 (nose)	6	D^2C^2 (nose)	2	D^2 (nose)	5	$D^2C^2C^1$ (nose)
										1	D^2 (throat)
E. V. E.	C^2	2	C^1C^2	3	C^2	1	C^2A^2	7	D^2		

CLINICAL CASES.*

M. P.	C^1D^2C	3	D^2C^1A	5	D^2C^1A	5	D^2C^1				
F. P.	D^2C^1	3	C^1D	5	D^2C^1	5	D^2				
P. C.	CDC^1	4	D^2								
O. C.	D^2C^1	2	D^2C^1	2	D^2C^1	2	D^2C^1A				

CONTACT CASES.

S. K.	D^2C^1	5	D^2DC^1	1	$C^1C^2D^2$	1	D^2C^1	6	D^2C^1D	4	D^2
Mr. P.	C^1C^2	1	CC^1C^2	1	C^1C^2D						
J. K.	D^2C^1D	2	D^2C^1	3	C^1C^2						
B. K.	D^2C^1	2	D^2C^1	3	D^2C^1	2	C^1D^2	2	D^2C^1	1	D^2C^1
W. D.	D^2C^1C	2	CC^1	3	C^1	2	D^2CC^1				
M. O.	CC^1D	7	C^1D^2								
A. D.	D^2C^1	2	D^2C^1	3	D^2						
Miss P.	D^2C^1	1	D^2C^1C	3	C^1C^2	1	C^1C^2				
A. C.	CC^1	2	D^2C^1	3	C^1CD						
J. K.	C^1D^2	1	C^1D^2	1	C^1D^2						

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* Examinations hereafter were made by Professor F. P. Gorham.

of weight. These signs were very transitory and it was found to be difficult to recover the organism if allowed to remain within the body for more than 18 to 24 hours. All but one of these strains produced some acidity in dextrose broth with phenolphthalein as an indicator, but the amount was so slight in each case that the reaction would have been alkaline to litmus, the indicator used by most observers.

TABLE 2.

No.	Source	Organism	Virulence	Isolated from:	Reaction in Dextrose
1	E. P.	B. hofmanni	Non-virulent	Diphtheria	Not tested
2	X.	"	"	"	" "
3	S. H.	"	"	Contact	0
4	M. W.	"	"	"	0.05
5	C. G.	"	"	S. F. Contact	0.15
6	J. DeZ.	"	"	" "	Not tested
7	I. M.	"	"	Diphtheria	" "
8	G. P.	"	"	Contact	0.15
9	A. S.	"	"	"	Not tested
10	E.	B. diphtheriae	"	"	1.05
11	McD.	"	Virulent	Diphtheria	0.50
12	B.	"	"	"	0.85
13	J. L.	"	"	"	0.53
14	S. S.	B. hofmanni	Non-virulent	Contact	0.05
15	E. S.	"	"	"	0.15
16	W. G.	"	"	"	0.20
17	M. T.	"	"	"	0.10
18	L. M.	"	"	"	0.05
19	E. B.	"	"	"	0.15
20	R. B.	"	"	"	0.15
21	E. U.	"	"	"	0.13
22	A. B.	"	"	"	0.20
23	P. V.	"	"	"	0.05
24	D. V.	"	"	"	0.15
25	E. C.	"	"	"	0.20
26	F. U.	"	"	"	0.10
27	T. P.	"	"	"	0.05
28	K. W.	B. diphtheriae	Virulent	Diphtheria	1.20
29	V. McA.	"	"	"	1.50
30	C. M.	"	"	"	1.30
31	XXXI	"	"	"	0.90
32	27y	"	"	"	Not tested

1. BY SUCCESSIVE PASSAGES THROUGH SUSCEPTIBLE ANIMALS.

Guinea-pigs.—Serial inoculations were tried with a number of strains of B. hofmanni. They were allowed to stay in the body of one guinea-pig as long as possible and still be recovered. Twenty-four hours, as mentioned above, was about the average length of time. The organism recovered was then grown in broth and again inoculated. Strain 1 was in this way passed through four guinea-pigs; No. 5, through ten; No. 6, through eight; and No. 8, through six. The result in all cases was the same. Occasional barred forms were observed but no permanent change was produced, the barred forms appearing in the culture recovered from one animal and not appearing in the succeeding culture at the time of inoculation or in the next culture recovered. The organisms were the same morphologically and were as non-virulent to guinea-pigs at the end as at the beginning of the series.

Canaries.—In carrying on inoculation experiments with canaries, two-fifths to one-half of a c.c. of a 48-hour culture was the amount generally used. The birds were Harz Mountain canaries weighing about 15 gm. each, the dose, therefore, being about three per cent of their body weight. Nine strains of B. hofmanni (Nos. 4, 5, 9, 15, 16, 18, 20, 22, and 25) were inoculated into as many canaries intramuscularly, all proving to be non-virulent. Some subcutaneous inoculations were tried with no very great success because of the extremely small amount of subcutaneous tissue and thin integument. When so inoculated, however, a very slight edema was produced. Several successive inoculations were made with No. 9 but it was found to be very difficult to recover the organism even if the canaries were killed in 18 hours. One inoculation of 1 c.c. (No. 16) was made, putting $\frac{1}{2}$ c.c. into each pectoral muscle. Even this large amount, about seven per cent of the body weight, produced no ill effect, while $\frac{1}{2}$ of a c.c. of a virulent diphtheria culture (Strain 13) killed a canary in 24 hours. In all, 15 canaries were inoculated with the various strains of B. hofmanni mentioned. Quite contrary to Salter's results, not one strain proved virulent to these birds.

Pigeons.—Five strains of the pseudodiphtheria bacillus (Nos. 1, 5, 6, 8, and 9) were inoculated into pigeons intramuscularly. Little if any edema was produced and it was found necessary to kill the pigeon in about 15 hours after inoculation in order to recover the organism. A short series of successive inoculations was made with strain No. 1, but it was evident that pigeons were even less susceptible than guinea-pigs to the action of B. hofmanni. Three strains of virulent diphtheria organisms (11, 12, and 13) were inoculated into as many pigeons. The one inoculated with No. 13 died in two days with marked whitening and degeneration of the muscle fibers. The other two pigeons were apparently not much affected by the dose which was over one per cent of their weight. As these two strains had killed guinea-pigs in two and three days respectively, it would seem that pigeons may be somewhat immune to diphtheria toxin.

Chickens.—Three strains of B. hofmanni (Nos. 5, 8, and 9) were inoculated intramuscularly. No ill effects were observed. In several subsequent inoculations it was found impossible to recover the organism even in 20 hours. Great care was taken, portions of the muscle being removed aseptically and incubated on blood-serum, but the organism had evidently disappeared. Virulent diphtheria organisms (Strain 11) were three times inoculated into chickens. The first died in sixteen days, the second in three days, and the third in four days. In all cases the muscle showed very marked degeneration, the tissues being yellowish white and friable, but even with great care only in the last case were the organisms recovered. Chickens are apparently not at all susceptible to the pseudodiphtheria bacillus and while they are evidently susceptible to the diphtheria toxin, the difficulty with which the organisms were recovered would seem to throw doubt upon the possibility of the transmission of the disease between fowls and humans.

2. BY THE INOCULATION OF LARGE DOSES AND BY THE USE OF IMMATURE ANIMALS.

Large amounts of broth cultures were next inoculated. No. 9 was passed successively through three guinea-pigs using 3½ per cent, 4 per cent, and 7 per cent of the body weight. Two guinea-pigs were inoculated with 2 per cent and 4 per cent respectively of No. 8. As diphtheria is so largely a disease of children, it was thought that very young guinea-pigs might be more susceptible. Accordingly several very small guinea-pigs were inoculated with doses as high as 7 per cent of their body weight. A large amount of edema was caused by all of these excessive inoculations. This generally extended over a large portion of the ventral surface of the body but apparently no effect other than this local disturbance was produced. The type of the organisms remained the same and the edema had disappeared by the end of 24 to 48 hours.

3. BY SENSITIZING CULTURES WITH HOMOLOGOUS SERUM.

According to White and Graham (1909), the infective power of the tubercle bacillus for rabbits was increased by sensitization of the organisms with normal rabbit serum. Their method was given a brief trial on the pseudodiphtheria bacillus. Blood was collected from guinea-pigs aseptically and the serum pipetted off. One blood-serum culture of Strain 16 was emulsified in 5 c.c. of this serum, incubated for 30 minutes, and inoculated into a guinea-pig. The same procedure was followed with Strain 22 with an incubation of 45 minutes. Neither inoculation had apparently any more effect on the guinea-pigs than when the organisms were not so treated.

4. BY GROWING CULTURES IN AN INCREASED SUPPLY OF OXYGEN.

It is well known that B. diphtheriae produces toxin more plentifully when there is a large supply of air. Presumably it is the oxygen of the air which is advantageous. It was thought that possibly an increased amount of oxygen might encourage the production of toxin by the pseudodiphtheria bacillus. This was accordingly tried.

Inoculated broth cultures were placed in a vacuum desiccator from which the air was exhausted by means of a water pump, until a pressure of about 12 m.m. was obtained. This was measured by a manometer and then a measured amount of oxygen was permitted to pass in. Three strains of B. hofmanni were incubated for three days with 40 per cent of oxygen, and three others with about 80 per cent. Guinea-pigs inoculated with the cultures so treated showed no unusual effects.

5. BY GROWING CULTURES IN CELLOIDIN SACS IN A SUCCESSION OF ANIMALS.

It was next determined to try a series of experiments using the well-known celloidin sac method, with the hope that a longer growth in the body of a susceptible host might tend to increase virulence. These sacs were made according to the usual technic with a few minor exceptions. It was found to be better not to fill the perforated glass tube with water in order to force off the capsule by the breath (Gorsline, 1903), but to draw it off by careful pressure and manipulation with the fingers, using the perforation at the end of the tube merely as a means for the ingress of air to prevent the collapse of the sac. The sacs were not placed in water at all until they had been constricted on to the end of the glass tube and were ready for sterilization. During the first experiments with this method, the capsules were incubated in sterile broth both before and after removal from the peritoneal cavity. In all cases, no growth was observed in the broth thus controlling the accuracy of the technic.

Strain 5 was passed through three successive guinea-pigs by this method. The first capsule remained in the peritoneal cavity 11 days. It was then removed, the organism recovered and placed in a new capsule which remained in a second pig for 14 days. This process was repeated in a third pig for 7 days, making a total of 32 days that this strain grew in the body of a susceptible animal. No effects were observed either on the pigs or on the organism. A few segmented forms were recovered from the second capsule but were not present in the culture recovered from the last.

It was thought that the insertion of a sac filled with the pseudodiphtheria bacilli together with a simultaneous peritoneal inoculation of virulent diphtheria organisms might produce the proper body fluids for the desired metamorphic change. In this way, the pseudodiphtheria culture would not be contaminated, nor be destroyed by the phagocytes. Both the diphtheria toxin and the body fluids of an animal suffering from the disease must necessarily come into contact with organisms so treated.

Strain 5, a pseudodiphtheria bacillus, and Strain 11, a virulent diphtheria bacillus, were accordingly inoculated in this manner. The pig died in two days with the characteristic symptoms of diphtheria poisoning but only D^2 forms were recovered from the capsule. It was attempted to prolong the time in which the pseudodiphtheria bacillus should remain bathed in these fluids by the insertions of two capsules, one containing B. diphtheriae and the other the pseudodiphtheria bacillus. Strains 5 and 11 were again used. The capsules were small, containing not much over ½ c. c. each. Altho the pig lost about 100 gm. in weight during the week following, it did not die. It was killed at the end of the week and organisms as typical as when they were inserted were recovered from the respective capsules. These recoveries were again placed in capsules and grown in the peritoneal cavity of another pig for seven days. The result was the same as before. Similar experiments were performed with Strains 3 and 11, pseudodiphtheria and true diphtheria cultures, respectively, passing the organism through two pigs, nine days in the first and eight in the second. Strain 5 was also tried again, using Auroccocus aureus in the other capsule to see what effect the symbiosis with that organism would have. The results of all the experiments were the same. The pseudodiphtheria bacillus still retained its usual characteristics.

6. BY INOCULATION OF ANIMALS IN COMBINATION WITH TOXIN, DIRECTLY OR IN CELLOIDIN SACS.

Believing that the presence of toxin might induce the pseudodiphtheria bacillus to become virulent, a celloidin capsule containing a broth culture of Strain 16 was inserted into the body cavity of a guinea-pig together with a peritoneal inoculation of 1 c.c. of sterile weak diphtheria toxin. This toxin had been obtained by the ordinary process of filtering a culture through a Pasteur filter. It had been proved sterile by incubation and by inoculation on blood-serum. Eighteen days later this pig was inoculated subcutaneously with $\frac{1}{4}$ c.c. of a more potent sterile diphtheria toxin, the minimum lethal dose of which was about 0.2 c.c. The pig died the next day with characteristic symptoms of diphtheria poisoning, and typical pseudodiphtheria bacilli with an occasional barred type were recovered from the capsule.

The same strain (16) was placed in a capsule in the peritoneal cavity of a guinea-pig together with a capsule containing the more potent toxin. The pig died in two days. D^2 was recovered.

Three capsules were placed in the body cavity of one pig, one capsule containing Strain 20; the second, Strain 16; and the third, toxin. The pig was killed and autopsied at the end of the fourth day. The adrenals appeared characteristic for diphtheria poisoning but typical B. hofmanni were recovered from the two capsules in which they had been placed.

A four-day culture of Strain 4 was inoculated together with $\frac{1}{4}$ c.c. of the toxin. The guinea-pig died in about 30 hours, the recovery showing typical D^2.

Strain 3 was inoculated together with $\frac{2}{5}$ c.c. of this toxin. The guinea-pig inoculated died in 24 hours. Two blood-serum cultures recovered from this pig were emulsified in dextrose-free broth and this was again inoculated together with $\frac{1}{5}$ c.c. of the toxin. Upon the death of the guinea-pig in 40 hours, the process was repeated with a third pig. The organisms recovered from all these inoculations were typical B. hofmanni.

7. BY A PROCESS OF SELECTION OF THOSE TYPES APPROACHING THE MORPHOLOGY OF VIRULENT ORGANISM FOR A SERIES OF "GENERATIONS."

Two strains of pseudodiphtheria bacilli were chosen, one, Strain 15, because it generally showed small organisms with blunt ends, and the other, Strain 16, because it usually displayed long forms with more or less pointed ends and a larger number of barred types than any other strain among those tested by the writer. In other words, Strain 15 looked the least like the true diphtheria bacillus and Strain 16 the most like that form.

These strains were streaked out on blood-serum, incubated, and the following day from 20 to 30 individual colonies were fished and inoculated on blood-serum tubes. After growth for slightly varying periods but usually about 16 to 20 hours, the cultures were examined microscopically and that one which possessed the largest types and segmented forms, if any were present, was selected and the process repeated. This was carried out 12 successive times with the following results.

Strain 15 occasionally showed longer forms but there was no continuous sequence. Strain 16 showed a few segmented forms in a large number of cultures throughout the series. They seemed to be more plentiful when the cultures happened to be incubated for a longer period than usual and also when there were only a few comparatively large colonies in a tube. These two conditions are similar, for in one we have the individual organism producing a large number of generations because of long-continued incubation, and in the other we have the same result because of more favorable conditions of growth.

The cultures from the seventh selection of Strain 16 were incubated two days and on examination one tube in particular, which had only a few large colonies, showed an unusual number of types resembling C^1 and A^1. The next lot of cultures from this selection were incubated about 20 hours and only one or two segmented types were observed in any of the smears. These cultures were replaced in the incubator and the growth continued up to 65 hours. A marked change of types had occurred in all of the tubes. Some of the tubes showed in the smears from 20 per cent to 40 per cent of barred types, many of them taking the stain rather poorly (Fig. 1).[1] The next series of cultures from the selection of this date, however, was incubated the usual length of time, and on careful examination, scarcely a barred form could be found. Strain 15, which was put through the same procedure, showed no such change of types during the long incubation but retained its usual appearance. At the end of the 12 selections, both strains presented the same morphology as originally and were still non-pathogenic to guinea-pigs.

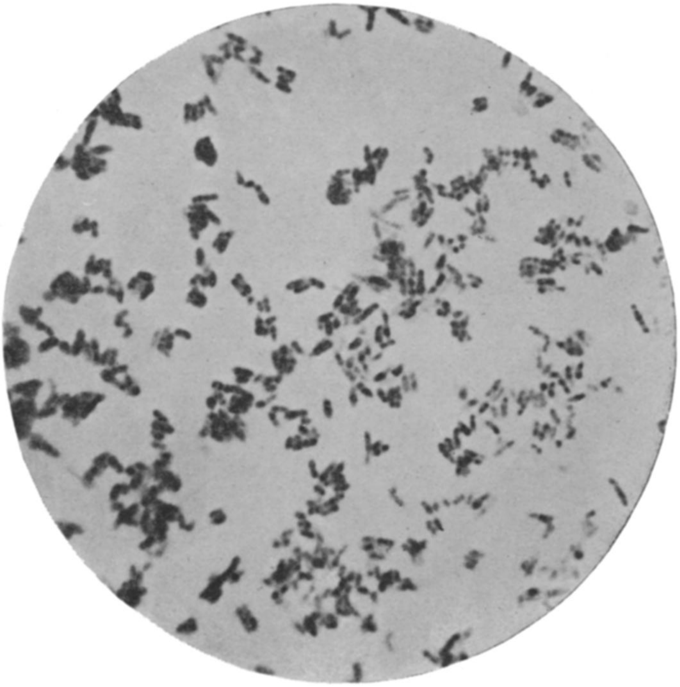

FIG. 1.—Strain No. 16. B. hofmanni. Showing barred and faintly staining involution types in a culture from the eighth selection grown 65 hours. ×2,000.

The results of this series together with a number of other minor observations led the writer to believe that possibly B. hofmanni might at times pass through a cycle of morphological changes similar to those described by Denny (1903) for B. xerosis and B. diphtheriae. Accordingly it was decided to repeat a portion of his work.

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[1] I am indebted to Dr. Edward Leaming for the photomicrographs.

From a study of the types of the organism appearing at different stages in the growth of cultures.—Four strains of B. pseudodiphtheriae (Nos. 3, 4, 16, and 19), three strains of virulent B. diphtheriae (Nos. 11, 12, and 13), and one strain (No. 10) of non-virulent B. diphtheriae were used. Denny's technic was followed carefully. Emulsions in broth were made from 24-hour cultures of these organisms and several loopfuls of this mixture smeared over the surface of moist blood-serum. The cultures were incubated at body temperature and smears made from each at the end of 4, 8, 12, 15, 27, 49, and 72 hours. Different parts of the surface of the serum were used for each preparation and the morphology of the organisms carefully noted.

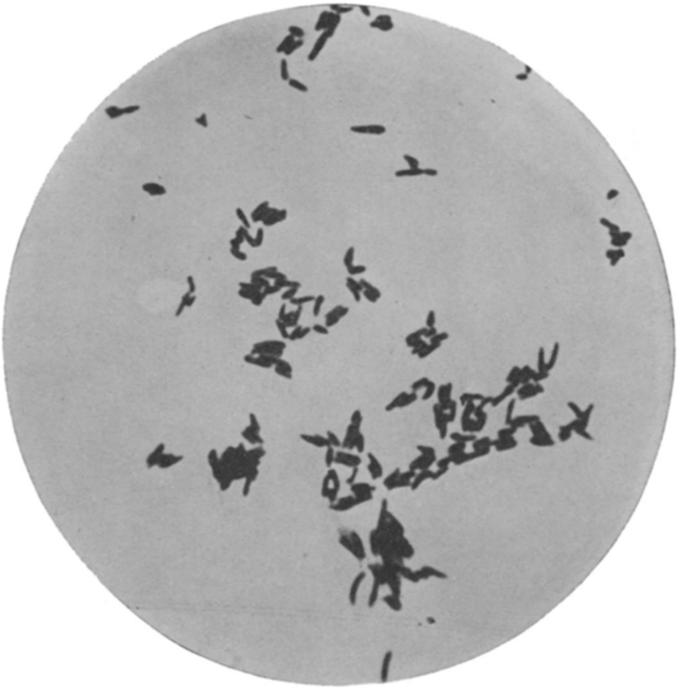

FIG. 2.—Strain No. 12. Virulent B. diphtheriae. Eight hours' growth. ×2 000.

The results differed from those obtained by Denny only in minor details.

Four-hour cultures.—No growth was visible but a number of bacilli were found in the microscopical preparations. These were more abundant in the diphtheria than in the pseudodiphtheria cultures. The organisms at this state were all solid-staining. The pseudodiphtheria bacilli were all typical D^2. In the diphtheria cultures, also, D^2 predominated with one or two C^2 forms in Strain 10 and many short thick, solid-staining clubs (A^2) particularly in Strain 12. The D^2 forms from the true diphtheria cultures were apparently a little more curved and pointed at the ends than those in the pseudodiphtheria preparations at this stage.

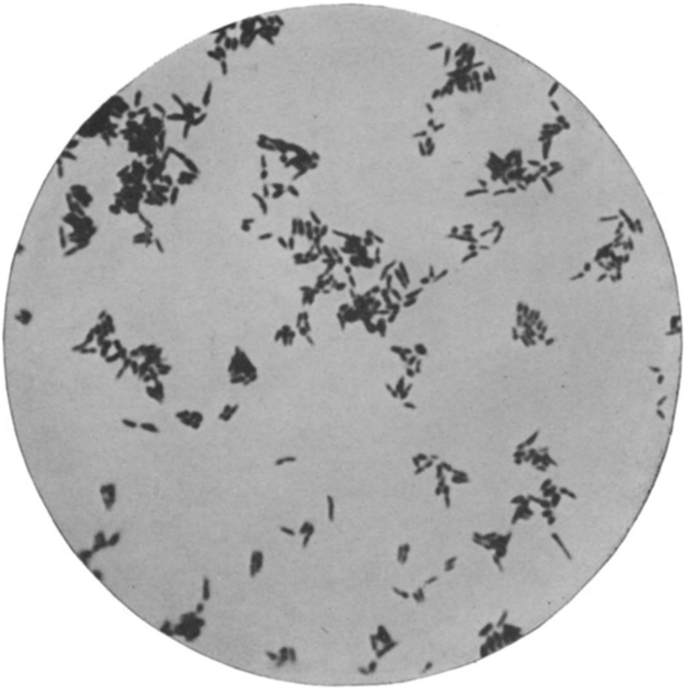

FIG. 3.—Strain No. 12. Virulent B. diphtheriae. Twelve hours' growth. ×2,000.

Eight-hour cultures.—The growth was barely visible on some of the tubes. The bacilli were all solid-staining, D^2 being the only type found in the pseudodiphtheria cultures. In the diphtheria cultures (see Fig. 2) the bacilli were longer, making a larger proportion of C^2 forms. Many of the D^2 forms were curved, some of them being thick and swollen either at the ends or in the middle. Fewer of the short A^2 types are present than in the four-hour stage.

Twelve-hour cultures.—The growth was distinctly visible. The bacilli from the pseudodiphtheria cultures were all of the D^2 type. Those from the diphtheria cultures (see Fig. 3) were longer with a number of C^1 types. There was still a large percentage of D^2 types, however.

Fifteen-hour cultures.—The bacilli from the pseudodiphtheria cultures were typical D^2. The diphtheria organisms (see Fig. 4) exhibited some granular types along with the C^1, C^2, D^2, etc.; D^2 was still present in considerable numbers, particularly in Strain 13.

Twenty-seven-hour cultures.—In this state of development, the pseudodiphtheria bacilli, with the exception of Strain 16, were noticeably shorter than in the earlier stages.

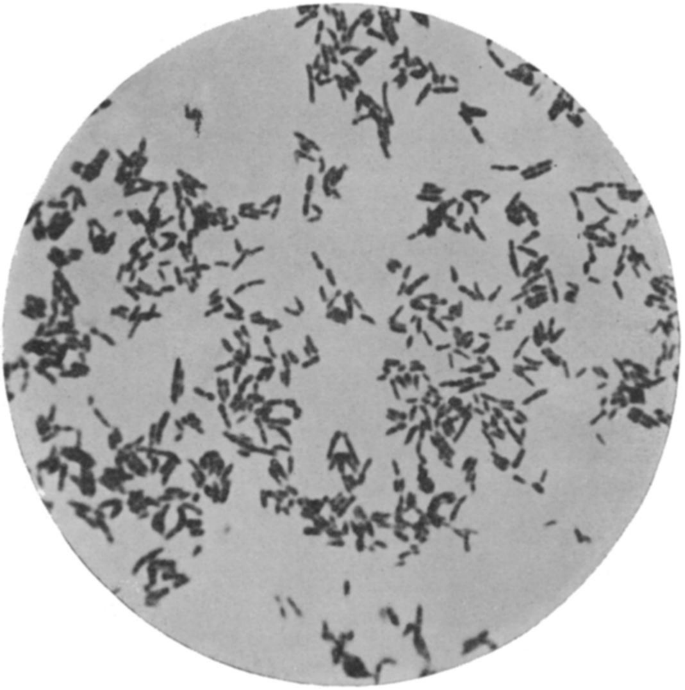

FIG. 4.—Strain No. 12. Virulent B. diphtheriae. Fifteen hours' growth. ×2,000.

The diphtheria cultures (see Fig. 5) contained more granular types. There was still a predominance of barred types, Strain 13 showing no granular forms whatsoever. D^2 was still present in three of the cultures, the only one in which it was not present being Strain 10, the non-virulent strain.

Forty-nine-hour cultures.—All but Strain 16 of the pseudodiphtheria strains displayed the short forms. In that culture the length of the earlier growths was retained and a few C^1 forms were observed. The B. diphtheriae showed no marked difference in morphology except that there were more granular forms. Many of the bacilli of both species took the stain poorly.

Seventy-two-hour cultures.—No particular differences were observed except that more organisms were faintly stained, more C^1 forms were observed in Strain 16, and a very few in Strain 21.

The results obtained coincided very exactly with Denny's except that the cultures as a whole seemed to develop a little more slowly than his. The solid-staining forms of the true diphtheria bacillus, comprising all of the organisms grown in eight-hour cultures and a considerable number in the cultures of longer incubation of some strains, would certainly be confused with the similar types of the pseudodiphtheria bacillus when found in throat or nose cultures. In these strains of the diphtheria bacilli, the D^2 types seemed to persist longer and there were more C^1 forms than in those observed by Denny. Also, it was found that a few C^1 forms were present in the older cultures of two strains of the pseudodiphtheria bacilli.

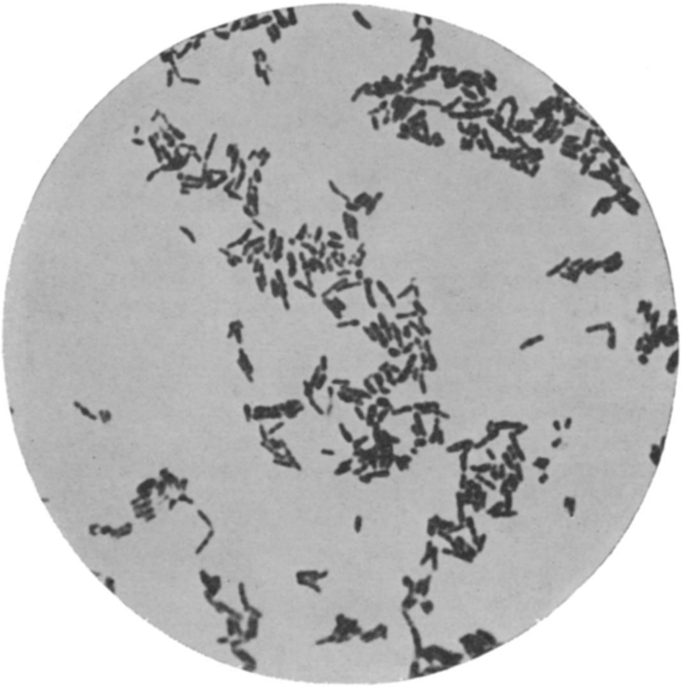

FIG. 5.—Strain No. 12. Virulent B. diphtheriae. Twenty-seven hours' growth. ×2,000.

In this connection, the laboratory history of Strains 31 and 32 are of interest. Strain 31 was isolated from a clinical diphtheria case and after incubation for about 16 hours showed a morphology so closely resembling that of B. hofmanni that it was so regarded.

This culture remained standing at room temperature for about two weeks. Upon re-examination, it still showed D^2 forms. It was grown in broth for 48 hours and inoculated into a rabbit, killing the animal in four days. Both the blood-serum culture from the broth at the time of inoculation and the culture recovered from the rabbit showed typical diphtheria organisms. Subcultures and pure cultures from the original culture, in all except one instance, showed typical diphtheria organisms. That one showed long D^2 forms after incubation for about 16 hours (see Fig. 6). On replacing this culture in the incubator, however, and growing for another 24 hours, the morphology became typical for B. diphtheriae.

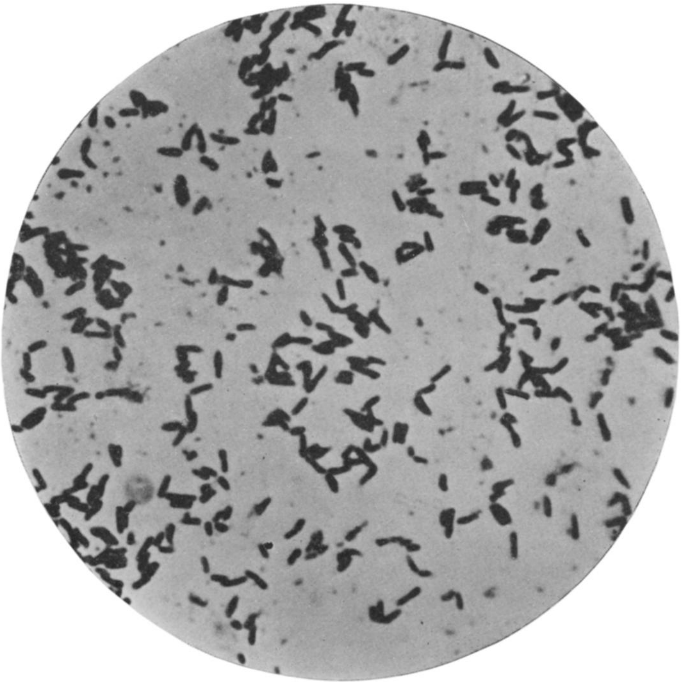

FIG. 6.—Strain 31. Virulent B. diphtheriae. Solid staining D^2 types in culture incubated for sixteen hours. ×2,000.

Strain 32 was isolated from a case of clinical diphtheria displaying large A, A^1, and C types. It was inoculated into a guinea-pig; the pig died; and the organisms recovered were typical D^2 types. The culture recovered from this guinea-pig was again inoculated into a guinea-pig, killing the pig in 35 hours with symptoms characteristic for diphtheria poisoning. $D,^2$ C^2 were the types recovered (see Fig. 7).

Still another item, in connection with morphological changes, may be worthy of comment. Two strains of B. hofmanni (8 and 9) were grown in large, tall blake bottles containing slants of Löffler's blood-serum, for one week at 37° C., and for five weeks at room temperature. Examinations at the end of that period showed large club and dumb-bell shaped forms with bars and granules much resembling similar types of B. diphtheriae. A 48-hour broth culture from the bottle culture of Strain 9 was inoculated into a guinea-pig. As the animal showed no symptoms of illness, it was killed the next day and typical B. hofmanni were recovered. Strain 8 immediately reverted to its original form on being inoculated on a fresh blood-serum tube.

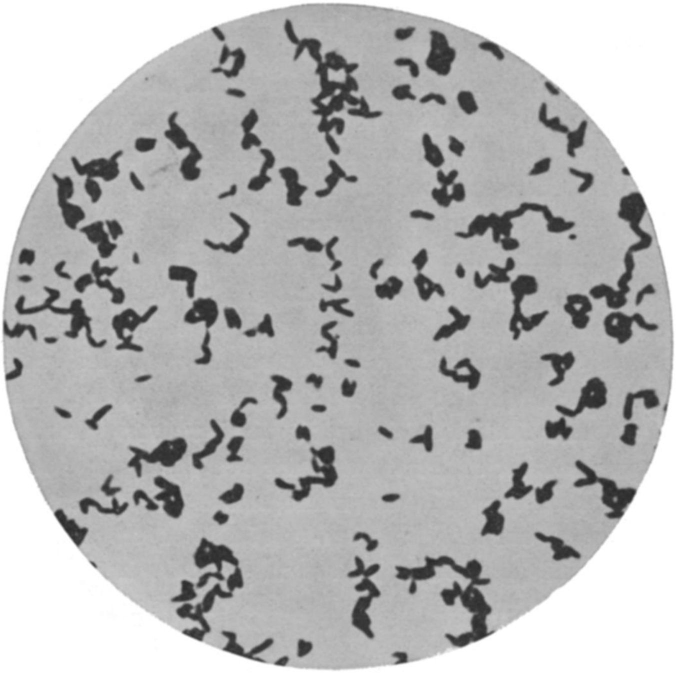

FIG. 7.—Strain No. 32. Virulent B. diphtheiiae. Solid staining types recovered from guinea-pig which showed characteristic symtoms of diphtheria poisoning. ×2,000.

From a study of the frequency curves of acid production of the pseudodiphtheria and diphtheria organisms.—The biometric method of studying variations having been so successfully used by Winslow (1908) in determining the systematic relationships among the Coccaceae, it was thought that that method might throw some light upon the conditions in this group. The action of the organisms on dextrose broth was chosen as being a character that was definitely measurable by titration and, therefore, best adapted for work of this sort.

One hundred and thirty-one pure cultures of the pseudodiphtheria bacillus obtained in carrying out the selection experiments described above, and 35 subcultures from the other strains tested in this investigation, were used in constructing the curve of B. pseudodiphtheriae. Sixty-two cultures of B. diphtheriae, mostly pure cultures from Strains 12, 13, and 28 and a few from the other strains, were used to determine the curve for that organism.

The cultures were all grown in 1 per cent dextrose broth and incubated at 37° C. for 72 hours. They were then boiled one minute to drive off the carbon dioxide and immediately titrated with N/20 NaOH, phenolphthalein being used as the indicator. In all cases a considerable number of blanks were run and the average subtracted from the titration figures to obtain the amount of acid actually produced by the organisms. The results of this experiment are given in Tables 3 and 4, arranged in ascending scales according to the amount of acid produced. Considerable variation will be noticed in the amount of acid produced by B. hofmanni but in no case is it in sufficient quantity to be acid to litmus, the indicator which has been used by most observers. Still wider variation is displayed by B. diphtheriae, many cultures not producing enough acid to turn litmus solution. This may be partly due to poor growth, altho Goodman (1908) obtained many results as low as these. The plotted frequency curves from these figures are shown in Chart 1.

The curves certainly display marked differences, the mode of one falling at +0.05 while that of the other falls at +0.90. The curve of the pseudodiphtheria bacillus is based upon enough figures from a sufficient number of strains to be fairly accurate. The curve of the true diphtheria organism, however, is not entirely satisfactory.

Because of the wide range of variation in the amount of acid produced by different strains of this organism, a satisfactory curve on this scale could not be produced from less than five or six hundred pure cultures, preferably from at least one hundred separate strains.

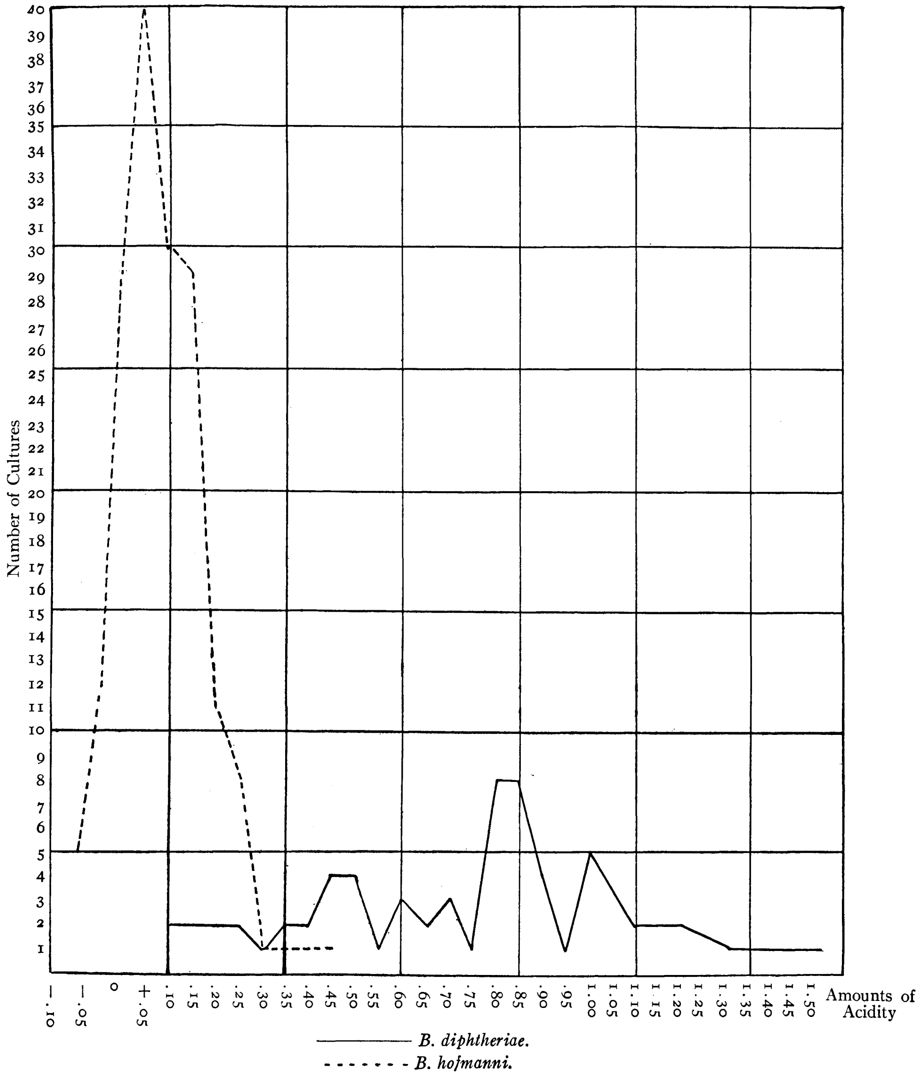

CHART 1.

The writer believes, however, that the curves here presented are sufficiently accurate to show that there is a marked difference between the two organisms.

TABLE 3.

Acid Production of B. hofmanni.

Acidity	No. of Cultures	Acidity	No. of Cultures	Acidity	No. of Cultures
−0.11	1	0.03	1	0.15	17
−0.09	2	0.05	19	0.16	10
−0.08	2	0.06	11	0.20	7
−0.05	2	0.07	9	0.21	4
−0.04	3	0.09	2	0.25	7
−0.03	7	0.10	14	0.26	1
0	12	0.11	10	0.32	1
0.01	6	0.12	4	0.45	1
0.02	11	0.13	2		

Total number of cultures, 166.

TABLE 4.

Acid Production of B. diphtheriae.

Acidity	No. of Cultures	Acidity	No. of Cultures	Acidity	No. of Cultures
0.12	2	0.50	1	0.87	7
0.25	1	0.52	1	0.90	2
0.27	1	0.53	1	0.92	2
0.30	1	0.62	3	0.97	1
0.35	1	0.65	1	1.00	5
0.37	1	0.67	1	1.10	2
0.42	2	0.72	3	1.15	2
0.43	1	0.77	1	1.20	2
0.45	2	0.78	1	1.30	1
0.47	1	0.82	7	1.37	1
0.48	2	0.85	1	1.50	1

Total number of cultures, 62.

SUMMARY OF THE ABOVE EVIDENCE.

We may sum up the evidence as follows: The series of cultures taken during the course of the disease show no tendency, except in a few cases, toward a change to the solid-staining forms. We believe, from our observation, that the sequence of types during the development of individual cultures, together with the fact brought out by Denny, that symbiosis with a large number of other organisms inhibits the change to granular types, adequately explains any opposite results.

The successive passage of a number of strains of B. hofmanni through many guinea-pigs, animals peculiarly susceptible to diphtheria toxin, did not change the morphology nor increase the virulence of the organisms. B. hofmanni also proved to be non-virulent to canaries, pigeons, and chickens, and successive inoculations through these birds produced no effect.

Doses as large as 7 per cent of the body weight inoculated into both half-grown and very young guinea-pigs produced no change in B. hofmanni, nor did these excessive inoculations have any other apparent effect on the animals than the production of a large amount of edema at the point of inoculation.

Cultures sensitized by contact with the serum of normal guinea-pigs produced, when inoculated, no additional effects on the guinea-pigs and no change in the organisms.

A large increase of the amount of oxygen present in the atmosphere in which the cultures of B. hofmanni were incubated did not produce any unusual effects.

Three passages through guinea-pigs, constituting a total stay of 32 days, in celloidin sacs in the body cavity of a susceptible animal, produced no change in B. hofmanni. Simultaneous inoculation with B. diphtheriae and with Aurococcus aureus, both with and without celloidin sacs, also caused no metamorphosis.

Cultures of B. hofmanni were inoculated into animals, together with diphtheria toxin, both directly and in celloidin sacs, with no change in the organisms inoculated.

A series of selections of those types approaching the morphology of virulent organisms made from cultures of B. hofmanni and carried through a period of twelve "generations," even in a strain which exhibited a number of barred types in the original culture, produced no permanent change. Each strain was as typical morphologically and in its action on guinea-pigs at the end as at the beginning of the experiment.

In individual cultures of B. diphtheriae, as described by Denny and confirmed by our own work, there occurs a sequence of types ranging from the solid-staining D^2, C^2 types through the barred to the granular types. We have even found one culture of virulent B. diphtheriae which presented only D^2 types even after 16 hours' incubation, and one culture, also, which exhibited D^2 types both before and after inoculation into a guinea-pig.

In considering the morphology, it should be further borne in mind that strains of B. hofmanni sometimes show occasional barred types, and in our observation, one culture of B. hofmanni, after 65 hours' incubation, showed a considerable proportion of these segmented types. Most strains, however, exhibit much smaller forms than normally, when incubated for this period.

Frequency curves of the acid production of the B. hofmanni and B. diphtheriae were markedly different. This seems to be positive evidence of specific difference between the two organisms.

DISCUSSION.

It seems to the writer that one reason for the confusion which has existed among bacteriologists in regard to these two species of bacteria may be traced to a misconception of the nature of species. There are always variations in a given species and merely because intermediate forms between one species and another can be found, it does not signify that the two are necessarily one. Particularly is this true in dealing with unicellular organisms like bacteria. We should naturally expect to find connecting forms between closely allied species.

Literally thousands of strains of pseudodiphtheria organisms have been tested for virulence only to find it lacking in all but possibly one or two cases. And as it is evident from our work that some strains of the true diphtheria organisms retain the solid-staining morphology much longer than is generally supposed, we think that most of these virulent D^2 types would have shown barred or granular morphology if they had been grown longer. If we could plot the curve of frequency of all these attempts conducted by many investigators in all parts of the world, it would present just the "mountain tops" and "valleys" we find in tabulating many admittedly different species. If only a hundred tests of the virulence of these organisms had been made, it would be the better part to suspend judgment in regard to this point until more data should be at hand. But as is well known, the number is not hundreds but thousands and is, therefore, sufficient basis for judgment.

As has been shown in this paper, the frequency curves of the action of the two organisms on dextrose are different. Here too, indeed, intermediate forms are found and some diphtheria organisms produce even less acid from dextrose than some pseudodiphtheria strains, but the modes of the curves are decidedly different.

In morphology, also, there is no question but that there is, as we should expect, an intergrading of types between the two species. There is also little doubt, however, that in well-grown cultures the two organisms usually show distinct morphological differences.

We think that a second very important cause for confusion lies in the failure to recognize that young forms of B. diphtheriae on blood-serum cannot be distinguished from the pseudodiphtheria bacillus. The morphology of the individuals of the two species should be compared only when they have been given proper opportunities for development.

The writer would suggest that a longer incubation than the customary 15 or 18 hours would probably in almost all cases settle the question as to the identity of a given bacillus. This would give opportunity for the D^2 forms of the diphtheria bacillus to develop into longer typical forms while the D^2 forms of the pseudodiphtheria bacillus would remain the same. It must be remembered, however, that *very long* incubation, 60 hours or more, may cause some strains of B. hofmanni to depart from the D^2 type. Also the virulent pseudodiphtheria types, which produce quite distinct symptoms from the true diphtheria bacillus, and whose toxic effect is not neutralized by diphtheria antitoxin, must be kept in mind.

Should not Wesbrook's symbol of D^2 be restricted to those organisms which produce acid and diphtheria toxin and never applied to the typical non-acid-producing, non-pathogenic pseudodiphtheria bacillus? The writer would also venture to suggest that this latter name be discarded for the less cumbersome and less perplexing one of B. hofmanni.

CONCLUSIONS.

1. Solid-staining types are not more prevalent at the end than at the beginning of a case of diphtheria.
2. Successive passages of B. hofmanni through guinea-pigs, chickens, pigeons, or canaries produce no effect either on the animals or on the organisms inoculated.
3. Doses as large as 7 per cent of the body weight of half-grown or young guinea-pigs do not kill the animals nor change the type of B. hofmanni.
4. Guinea-pigs inoculated with cultures of B. hofmanni sensitized with homologous serum show no unusual effects.

5. B. hofmanni grown in an increased supply of oxygen shows no biochemical or morphological change.

6. By using celloidin sacs it was found that long-continued growth in the body cavity of guinea-pigs either alone or together with B. diphtheriae or Aurococcus aureus does not change B. hofmanni.

7. B. hofmanni, inoculated into animals in combination with toxin, either directly or in celloidin sacs, exhibits no change in the cultures recovered.

8. Artificial selection on the basis of morphology does not change the form of B. hofmanni.

9. Solid-staining forms are common to both B. hofmanni and B. diphtheriae during the first 8 to 12 hours of growth. Occasionally, however, these types are retained by the B. diphtheriae for much longer periods and some strains of B. hofmanni may show barred types on long incubation.

10. The frequency curves of acid production of B. hofmanni and B. diphtheriae show marked differences.

11. We would suggest that the term pseudodiphtheria bacillus be discarded for the less perplexing one of B. hofmanni and that the symbol D^2 be restricted to those organisms of the correct morphology which produce acid and diphtheria toxin.

12. From a careful study of the literature and from the experiments described in this paper, we are forced to take the position that the pseudodiphtheria bacillus or B. hofmanni belongs to a different species from the true Klebs-Löffler bacillus. Doubtless both organisms do belong to the same group and came from common ancestors, but the differences seem to be sufficiently constant to place them in separate species.

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